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13. ABSTRACT ***(Maximum 200 Words)***

BI-1 is an integral membrane protein that protects cells against Bax-induced cell death. It is commonly expressed in prostate cancer cell lines, making it a candidate regulator of prostate cancer cell death. I have used genetics, biochemical and cell biology approaches to understand how BI-1 regulates apoptosis. I have cloned BI-1 homologs from yeast, bacteria, Drosophila and plants, and demonstrated that they function similarly in yeast to protect Bax-induced cell death; I have discovered that the yeast BI-1 homolog is important in protecting cells against heat stress. I have also determined BI-1 domains essential for its function and proper sub-cellular targeting. BI-1 K/O mouse are also underway and characterization of these animals will reveal BI-1's in vivo functions.

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INTRODUCTION

BI-1 is a novel anti-apoptotic regulator identified in our lab using a Bax-suppressor screen conducted in yeast (Xu & Reed, 1998). Its anti-apoptotic function makes it a candidate regulator of cancer pathogenesis or progression. BI-1 is expressed in all prostate cancer cell lines tested to date, thus making it a potential target for prostate cancer therapy. We have proposed to use genetics, biochemical and cell biology approaches to study the structure and function of this novel apoptotic regulator and its potential relevance to prostate cancers.

BODY: The accomplishments in each task are summarized below:

Task 1. To compare the structure-function activity of human and yeast BI-1. This task was successfully accomplished. I have cloned yeast BI-1 and showed that over-expression of yeast BI-1 functions in yeast to protect against Bax-induced cell death. A yeast strain with deletion of the yeast BI-1 gene was also obtained and found more susceptible to cell death induced by heat stress, indicating that yBI-1 is important in mediating protection against stress conditions. However, Bcl-2 and BI-1 failed to rescue the yBI-1 deletion strain from its sensitivity to heat-induced cell death, even though they can rescue the yBI-1 deletion strain from Bax-induced cell death. Attempts to study yBI-1 in mammalian cells were unsuccessful due to poor expression (probably caused by differences in codon bias). Expression level of yBI-1 in mammalian cells is very low based on Western blotting analysis.

Task 2. To determine how BI-1 suppress Bax-induced cell death in mammalian cells. I have struggled with this task due to poor signal to noise ratios in cell death assays involving transient transfection of BI-1 into mammalian cells. Initial attempts to produce stable transfectants of BI-1 were also unsuccessful. Goals for the upcoming year are to try transient and stable expression of BI-1 in different cell lines, since cell context may be important determinant of stability of the BI-1 protein or mRNA.

An alternative strategy for determining BI-1's function is to generate BI-1 knockout mice and determine their phenotype. We identified an ES cell in which the BI-1 gene has been disrupted by retrovirus insertion (www.lexgen.com). These ES cells have been used to generate heterozygous BI-1 knockout mice, and we are mating these animals to generate homozygous knockouts. The phenotype of the mice and their susceptibility to various apoptotic stimuli will be determined. This will ultimately reveal BI-1's in vivo function.

Task 3. To determine the significance of BI-1's interaction with Bcl-2 and Bcl-Xl. Mutants of BI-1 have been generated that failed to protect against Bax-induced cell death in yeast. I plan to test whether these mutants are capable of interacting with Bcl-2/Bcl-XL.

Task 4. Elucidate the structural features of BI-1 required for its cytoprotective function. This task was mostly accomplished. I have divided the BI-1 protein into 13 regions: N (N terminus), TM1 (transmembrane 1), TM1-2 (between transmembrane 1 and 2), TM2, TM2-3, TM3, TM3-4, TM4, TM4-5, TM5, TM5-6-1, TM5-6-2, TM6, C (C terminus). BI-1 mutants were made so that each construct has one deletion in one of

these regions. These BI-1 mutants were tested for their protection against Bax-induced cell death in yeast. Interestingly, most of these constructs failed to protect Bax-induced cell death, while only the constructs with deletion in the N, and TM1-2 were still able to rescue yeast cells. Site-directed mutagenesis was also used to change to alanine the c-terminal nine amino acids, which were mostly consisted of charged amino acids (C9A). The C9A mutants also failed to protect Bax-induced cell death. Furthermore, the subcellular localization of the BI-1-C9A seems to be altered based on fluorescence microscopy studies. Thus, the c-terminus may be important for both BI-1's function and localization.

Besides using the above deletion analysis, I also obtained BI-1 homologs from Arabidopsis and rice, putative BI-1 homologs from yeast and bacteria, BI-1 homologs from tomato and Drosophila through library screening. All of them can protect Bax-induced cell death in yeast. Sequence alignment of the human, Drosophila and plant BI-1 homologs also indicated that N-terminus is less conserved compared to other regions of the protein, and is therefore consistent with my results with the deletion analysis.

Unfortunately, none of these mutant BI-1 constructs or BI-1 homologs could be expressed at high levels in mammalian cells. Thus, it was not possible to ask whether they protect against Bax-induced apoptosis in mammalian cells. Further analysis with different approaches or different assays will be needed to fully understand BI-1's structural and functional relationship.

Task 5. Identify other proteins involved in the BI-1 regulated PCD pathway. This task was not accomplished since the method we planned to use for cloning BI-1 interacting proteins (split ubiquitin complementation method) did not work well and gave too much background.

Task 6. Determine the incidence of BI-1 expression in prostate cancers. This task was not accomplished due to the difficulties with generating BI-1 antibodies. We have raised three rabbit polyclonal antibodies against either BI-1 peptides or BI-1 recombinant protein. Unfortunately, none of them was very specific to BI-1 protein and all gave exceptionally high background. Therefore, expression analysis of BI-1 protein in prostate cancer cells was not carried out. Recently, two more rabbit polyclonal antibodies are being generated against BI-1 peptides and they will be tested soon.

Bcl-B, a novel anti-apoptotic Bcl-2 family protein. Besides working on BI-1 genes, I also cloned and characterized a novel anti-apoptotic Bcl-2 family protein, Bcl-B (Ke, et al, JBC, attached). Bcl-B is a 204 amino acid protein. It contains BH1, BH2, BH3, BH4 domains and a TM domain. It is mostly homologous to the mouse Boo/Diva gene. It is expressed in most tissues, including prostate. Expression of Bcl-B is able to protect against Bax-induced cell death. Bcl-B can also protect cells against death stimuli such as staurosporin and ultraviolet light. However, Bcl-B cannot protect against Bak-induced cell death. The difference between Bcl-B's protection against Bax and Bak-induced apoptosis can be explained by Bcl-B's interaction with Bax but not Bak. Bcl-B is localized to mitochondria, and the TM domain is important for both its localization and protective function. Rabbit polyclonal antiserum has been raised against a Bcl-B peptide.

Preliminary results indicate that Bcl-B is expressed in many cancer cell lines, thus making it a candidate regulator of tumor pathogenesis or progression.

Key research accomplishments:

- Demonstrated yeast BI-1 orthologue's functions in resistance to stress-induced cell death.
- Demonstrated that several BI-1 homologs from diverse organisms function in protecting yeast cells against Bax-induced cell death.
- Identified domains essential for BI-1's cyto-protective function.
- Cloned and characterized a novel anti-apoptotic Bcl-2 family protein, Bcl-B.

Reportable outcomes:

- A manuscript describing Bcl-B was published in JBC (Ke, et al, 2001).
- A patent application was submitted and pending for Bcl-B.
- A manuscript is in preparation describing the cloning of BI-1 homologs.
- BI-1 knock-out mice have been generated which provides resource needed for understanding BI-1's function.

Conclusions:

In conclusion, I have made substantial progress toward understanding the mechanisms by which BI-1 exerts its cyto-protective function: namely, the identification of BI-1 homologs from various organisms and the demonstration of their similar protective functions; and the identification of domains within BI-1 essential for its function. These findings provide the foundation for future studies. The mouse knock-out study will definitely demonstrate the in vivo function of BI-1, and will provide an animal model for future studies of BI-1's role in prostate cancer by mating mice with transgenic lines overexpressing oncogenes in the prostate. These studies will help us better understand BI-1's function and its possible role in prostate cancers.

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Xu,Q., & Reed, J.C. (1998). Bax inhibitor-1, a mammalian apoptosis suppressor identified by functional screening in yeast. Mol. Cell 1, 337-346.

Ke, N., Godzik A., and Reed, J.C. (2001). Bcl-B, a novel Bcl-2 family member that differentially binds and regulates Bax and Bak, J. Biol. Chem. 276, 12481-4.

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Bcl-B, a Novel Bcl-2 Family Member That Differentially Binds and Regulates Bax and Bak*

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A novel human member of the Bcl-2 family was identified, Bcl-B, which is closest in amino acid sequence homology to the Boo (Diva) protein. The Bcl-B protein contains four Bcl-2 homology (BH) domains (BH1, BH2, BH3, BH4) and a predicted carboxyl-terminal transmembrane (TM) domain. The *BCL-B* mRNA is widely expressed in adult human tissues. The Bcl-B protein binds Bcl-2, Bcl-X_L, and Bax but not Bak. In transient transfection assays, Bcl-B suppresses apoptosis induced by Bax but not Bak. Deletion of the TM domain of Bcl-B impairs its association with intracellular organelles and diminishes its anti-apoptotic function. Bcl-B thus displays a unique pattern of selectivity for binding and regulating the function of other members of the Bcl-2 family.

Bcl-2 family proteins play a central role in apoptosis regulation in metazoan species. In humans, over 20 members of this family have been identified to date, including proteins that suppress (Bcl-2, Bcl-X_L, Mcl-1, Bfl-1/A1, Bcl-W) and proteins that promote (Bax, Bak, Bok, Bad, Bid, Bik, Bim, Nip3, Nix) cell death (reviewed in Refs. 1 and 2). Bcl-2 family proteins contain at least one of four conserved regions, termed Bcl-2 homology (BH)[1] domains. Most members of this family also contain a TM domain located near their carboxyl terminus that anchors them in intracellular membranes of mitochondria and other organelles (reviewed in Refs. 1 and 2).

Many Bcl-2 family proteins are capable of physically interacting, forming homo- or heterodimers, and functioning as agonists or antagonists of each other (1–3). Specificity for interaction partners and tissue-specific patterns of expression combine to endow each mammalian Bcl-2 family protein with a unique physiological role *in vivo*, resulting for example in highly diverse phenotypes when members of this multigene family are individually knocked out in mice (reviewed in Ref. 4). Thus, a need exists to identify comprehensively the members of the Bcl-2 family and to elucidate their functional characteristics. In this report, we describe the molecular cloning and initial characterization of a new human member of the Bcl-2 family, Bcl-B.

MATERIALS AND METHODS

Cloning of BCL-B cDNAs—TBLASTN searches of the human expressed sequence tag (EST) data base using the amino acid sequence of the mouse Boo/Diva as a query resulted in the identification of homologous partial cDNAs. A human EST clone (GenBank™ accession number AA098865) was obtained (Research Genetics) and sequenced in its entirety, revealing an open reading frame (ORF) encompassing the last 151 residues of a protein with homology to Boo (Bcl-B) (submitted to GenBank™ with accession number AF326964). The corresponding genomic sequence for this cDNA was identified in the human genome data base (clone CTD-2184D3), which was derived from human chromosome 15q21. Because the EST clone lacked a candidate start codon, the corresponding 5′-end of Bcl-B cDNAs was cloned by a reverse transcriptase polymerase chain reaction (RT-PCR) approach, using the forward primer NKO118 (5′-CGGGCCAAGAAAACCAGCGAAGG-3′), which was designed to hybridize to a region upstream of the Bcl-B ORF as predicted from the genomic data, and the reverse primer NKO121 (5′-CACTCAAGGAAGAGCCATTTGCAT-3′), which is complementary to a region downstream of the predicted Bcl-B ORF corresponding to the 3′-untranslated region of the putative mRNA. PCR amplification using human liver cDNA (CLONTECH) as a template with the above primers yielded a single ~0.9-kb product, which was cloned into pCR2.1-TOPO (Invitrogen, following the manufacturer's instructions) to generate TOPO-Bcl-B (pNK254) and sequenced.

RT-PCR Analysis—Expression of *BCL-B* mRNA in various tissues was examined by RT-PCR, using oligo(dT)-primed first-strand cDNA derived from multiple adult human tissues (CLONTECH) as templates. cDNAs were amplified following the manufacturer's instructions using the forward primer NKO120 (5′-GTGGTGACGCTCGTGACCTTCG-3′) and NKO121 as the reverse primer. Glyceraldehyde-3-phosphate dehydrogenase primers were used as a positive control (5).

Plasmid Construction—The ORF encoding Bcl-B was PCR-amplified from TOPO-Bcl-B (pNK254) using the forward primer NKO101 (5′-GGAATTCATGGTTGACCAGTTGCGGGAG-3′) and reverse primer NKO103 (5′-CCGCTCGAGTCATAATAATCGTGTCCAGAG-3′). The PCR products were digested with *Eco*RI and *Xho*I and cloned into the *Eco*RI and *Xho*I sites of pcDNA3-Myc (Stratagene), and the *Eco*RI and *Sal*I sites of pcI-Neo-FLAG (Invitrogen) and pEGFP-C2 (CLONTECH). A plasmid encoding Bcl-B lacking its COOH-terminal transmembrane domain (Bcl-BΔTM) was constructed by PCR-based mutagenesis using primers NKO101 and NKO131 (5′-CCGCTCGAGTCATGTTTTCTCCAAAAAGCCAGTG-3′). The resulting PCR product was digested with *Eco*RI and *Xho*I and cloned into pcDNA3-Myc.

Cell Culture, Transfection, and Apoptosis Assays—HEK293, COS7, HT1080, and PPC1 cells were maintained in Dulbecco's modified Eagle's medium (Irvine Scientific) supplemented with 10% fetal bovine serum, 1 mM L-glutamine, and antibiotics. For transient-transfection apoptosis assays, cells (5×10^5) in six-well dishes were co-transfected using Superfect (Qiagen) with 0.5 μg of pcDNA3-Bax plus 0.5 μg of green fluorescence protein (GFP) marker plasmid pEGFP (CLONTECH) or 0.5 μg of pEGFP-Bak, and 1 μg of pcDNA3, pcDNA3-Myc-Bcl-B, pcDNA3-Myc-Bcl-BΔTM, or pcDNA3-FLAG-Bcl-X_L. The total amount of DNA was normalized to 3 μg per transfection using pcDNA3. At 24 h post-transfection, both adherent and floating cells were collected, fixed, and stained with 0.1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI). The percentages of apoptotic cells were determined by counting the GFP-positive cells having nuclear fragmentation and/or chromatin condensation (mean ± S.D.; $n = 3$).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF326964.

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[1] The abbreviations used are: BH, Bcl-2 homology; TM, transmembrane; EST, expressed sequence tag; ORF, open reading frame; RT-PCR, reverse transcriptase polymerase chain reaction; kb, kilobase pair(s); GFP, green fluorescence protein; DAPI, 4′,6-diamidino-2-phenylindole; HM, heavy membrane; LM, light membrane; PAGE, polyacrylamide gel electrophoresis; STS, staurosporine; hu, human; HA, hemagglutinin; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

For stable transfections, HeLa cells in 100-mm dish were transfected with pcDNA3 (control), pcDNA3-Myc-Bcl-B, or pRC-CMV-Bcl-2 plasmids using LipofectAMINE plus (Life Technologies, Inc.). Two days later, complete medium containing G418 (800 μg/ml) (Omega Scientific Inc.) was used to select stably transfected cells. Several of the resulting G418-resistant clones were recovered using cloning cylinders and individually expanded. G418-resistant clones were screened for the expression of desired genes by immunoblotting with antibodies. For apoptosis assays, stably transfected clones (5×10^5 cells) in six-well dishes (30 mm diameter) were cultured in medium containing various concentrations of staurosporine (Calbiochem) (0.2–1 μM) or of recombinant TRAIL (Biomol) (10–100 ng/ml) for 8–10 h. Both floating and adherent cells were collected, fixed, and subjected to DAPI staining, enumerating the percentage apoptosis cells by UV microscopy.

Immunofluorescence and Subcellular Fractionation—The intracellular location of Bcl-B was examined using fluorescence confocal microscopy and subcellular fractionation methods, essentially as described (6, 7).

Co-immunoprecipitation and Immunoblotting Assays—293T cells (5×10^5) cultured with 50 μM benzoyl-Val-Ala-Asp-fluoromethylketone (Bachem) were co-transfected with 1.5 μg of pcDNA3-Myc-Bcl-B, pcI-Neo-FLAG-Bcl-B, pcDNA3-human calcyclin-binding protein (used as a control), or pcDNA3-FLAG-Bcl-X_L, together with 1.5 μg of pEGFP, pEGFP-Bcl-B, pcDNA3-HA-BAG1, pcDNA3-HA-Bax, pcDNA3-FLAG-Bcl-X_L, pRC-CMV-Bcl-2, or pEGFP-Bak. At 24-h post-transfection, cells were collected and resuspended in lysis buffer (142.4 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES (pH 7.4), 0.5 mM EGTA, 0.2% Nonidet P-40) containing 12.5 mM β-glycerophosphate, 2 mM NaF, 1 mM Na_3VO_4, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture (Roche Molecular Biochemicals). Soluble lysates were incubated with 10 μl of anti-Myc (Santa Cruz) or anti-FLAG (Sigma) antibody-conjugated Sepharose beads overnight at 4 °C. Beads were then washed four times in 1.5 ml of lysis buffer and boiled in Laemmli gel-loading solution before performing SDS-PAGE/immunoblotting using the following polyclonal or monoclonal antibodies: polyclonal rabbit anti-GFP (Roche Molecular Biochemicals), monoclonal rat anti-HA (Roche Molecular Biochemicals), monoclonal mouse anti-FLAG (Sigma), monoclonal mouse anti-Myc (Santa Cruz), rabbit anti-huBcl-2, rabbit anti-hu Bcl-X_L, rabbit anti-hu Bax, or rabbit anti-hu Bak (8).

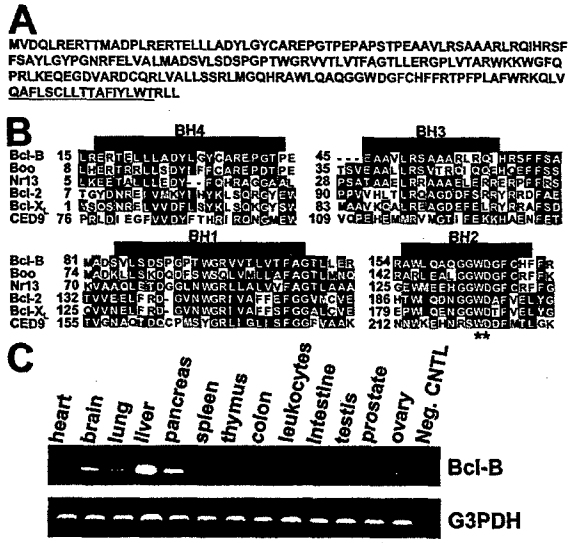

FIG. 1. **Sequence analysis and the expression of Bcl-B cDNA.** *A*, the predicted Bcl-B amino acid sequence is presented with the TM *underlined* (GenBank™ accession number AP326964). *B*, alignments of BH1, BH2, BH3, and BH4 domains of Bcl-B and other Bcl-2 family proteins are shown. *Numbers* on the *left* indicate the position of the amino acid in each protein based on GenBank™ accession numbers AAD08703 (murine Boo), Q90343 (chicken Nr13), AAA35591 (hu Bcl-2), CAA80661 (hu Bcl-XL), and P41958 (*C. elegans* CED9). Identical and similar residues are indicated by *black* and *gray boxes*, respectively. *Asterisks* under the BH2 alignment indicate the intron junction for hu *BCL-B*, *BCL-2*, and *BCL-X* genes. *C*, expression of *BCL-B* in adult human tissues. First-strand cDNAs made from RNA samples from various adult human tissues were PCR-amplified using *BCL-B*-specific primers. The reverse primer was downstream of the intron, thus avoiding amplification of contaminating genomic DNA. PCR products were size-fractionated by electrophoresis in 1% agarose gels, stained with ethidium bromide, and photographed under UV illumination. Primers specific for glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) were also used for PCR as a positive control.

RESULTS

During TBLASTN searches of the publicly available EST data bases using the amino acid sequence of the mouse Boo/Diva as a query, we discovered an EST clone (GenBank™ accession number AA098865) encoding a predicted polypeptide harboring a BH1 domain. PCR methods were used to obtain cDNAs containing the complete ORF corresponding to a 204-amino acid protein (Fig. 1*A*). The predicted ORF was initiated by an AUG start codon within a favorable Kozak context. The predicted protein contains regions resembling the BH1, BH2, BH3, and BH4 domains typical of anti-apoptotic members of the Bcl-2 family, as well as a putative carboxyl-terminal TM domain (Fig. 1*B*). Comparisons of the sequence of this predicted protein with all known Bcl-2 family members by BLAST search indicated that it is most similar to the murine Bcl-2 family protein Boo (also known as Diva) (9, 10), sharing 47% amino acid sequence identity, and thus prompting the moniker "Bcl-2 family protein resembling Boo" (Bcl-B). The *BCL-B* gene is located on chromosome 15 (map 15q21), as determined by in silico screening of the human genome data base at NCBI. Comparison of the *BCL-B* cDNA sequence with genomic data indicates a two-exon structure, in which the region encoding residues Trp^{163} and Asp^{164} (within the BH2 domain) of the Bcl-B protein are interrupted by an ~2.3-kb intron. PCR analysis suggested that the *BCL-B* mRNA is widely expressed in adult human tissues (Fig. 1*C*).

The Bcl-B protein was tested for interactions with other Bcl-2 family proteins by co-immunoprecipitation experiments, wherein Bcl-B was expressed in HEK293T or HT1080 cells with various NH_2-terminal epitope tags. These studies indicated that Bcl-B is capable of associating with itself, Bax, Bcl-2, and Bcl-X_L, but not with Bak (Fig. 2).

The function of the Bcl-B protein was explored by transient transfection in a variety of cell lines, including HEK293T, COS7, HT1080, and PPC1. Overexpression of Bcl-B did not induce apoptosis, nor did it negate suppression of apoptosis caused by overexpression of Bcl-2 or Bcl-X_L (not shown), suggesting that Bcl-B is not a pro-apoptotic protein. We therefore tested the possibility that Bcl-B is a cytoprotective protein by ascertaining its effects on apoptosis induced by the pro-apoptotic proteins Bax and Bak. Co-expressing Bcl-B markedly suppressed apoptosis induced by Bax but not Bak (Fig. 3), thus correlating with protein binding data demonstrating that Bcl-B associates with Bax but not Bak (Fig. 2). This suppression was not due to reduced levels of Bax protein, as determined by immunoblotting. In contrast to Bcl-B, co-expression of Bcl-X_L suppressed apoptosis induced by either Bax or Bak (Fig. 3).

To further explore the effects of Bcl-B on apoptosis, HeLa cells were stably transfected with a plasmid encoding Myc-tagged Bcl-B, *versus* control (empty) plasmid. Several stably transfected clones were tested for Bcl-B expression by immunoblotting, and their responses to apoptosis induced by staurosporine (STS) or TRAIL were compared. Comparisons were also made to HeLa cells stably transfected with a Bcl-2-encoding plasmid. Fig. 3, *C—E*, show representative results, where control transfected (vector) cells were compared with two Bcl-B-transfected clones. The Bcl-B-expressing clones shown here (clones 9 and 16) produced different relative amounts of Myc-Bcl-B protein, as determined by immunoblotting, with clone 16 containing ~5 times higher levels of Bcl-B than clone 9. HeLa cell clones such as clone 16, which contained higher amounts of Myc-Bcl-B, displayed resistance to apoptosis induced by STS and TRAIL, compared with control (vector)-transfected cells. In contrast, HeLa cell clones such as clone 9, which contained

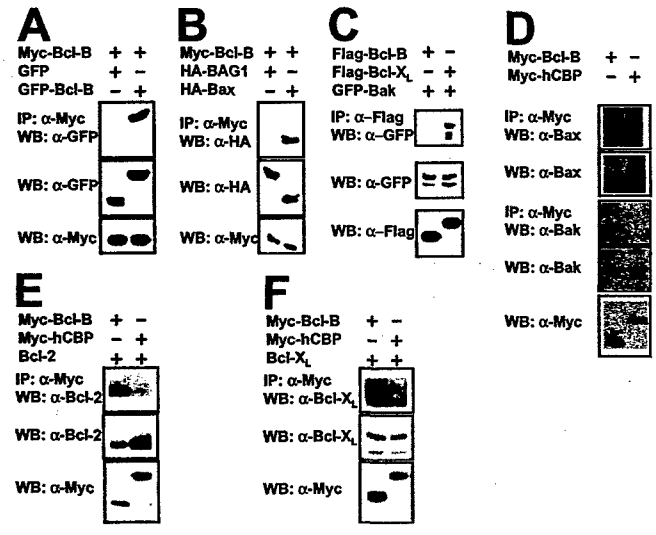

FIG. 2. **Bcl-B interacts with itself and select Bcl-2 family proteins.** HEK293T cells were transiently transfected with various combinations of plasmids encoding Myc-Bcl-B, Myc-human calcyclin-binding protein, GFP, GFP-Bcl-B, GFP-Bak, Bcl-2, FLAG-Bcl-X_L, HA-Bax, HA-BAG1, and FLAG-Bcl-B. Cell lysates were prepared and immunoprecipitated as described under "Materials and Methods." Lysates were prepared from equivalent numbers of cells, and immunoprecipitations (*IP*) were performed using either anti-Myc or anti-FLAG monoclonal antibodies (*top panel*), followed by SDS-PAGE/immunoblot analysis (Western blotting (*WB*)) using rabbit polyclonal antibodies specific for GFP (*A*, *C*), Bcl-X_L (*F*), Bcl-2 (*E*), Bax (*D*), or Bak (*D*) or rat monoclonal antibody specific for the HA tag (B). To verify expression of all proteins, equivalent volumes of lysates were also loaded directly in gels and analyzed by SDS-PAGE/immunoblotting (*WB*) (*middle* and *bottom panels*) using antibodies specific for GFP, HA, FLAG, Myc, Bcl-X_L, Bcl-2, Bax, or Bak. For efficiency of presentation, only the portion of the gels containing the relevant bands is shown. Additional controls, including immunoprecipitations using negative control antibodies, are also not presented in the figure. Note in *D* that interaction of Myc-Bcl-B with endogenous Bax but not endogenous Bak is demonstrated.

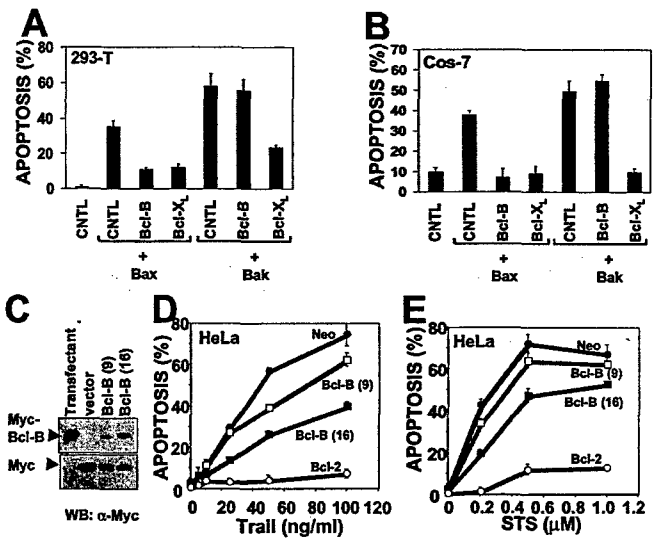

FIG. 3. **Bcl-B inhibits Bax- but not Bak-induced apoptosis.** HEK293T (*A*) and COS7 (*B*) cells at ~50% confluence in six-well dishes were co-transfected with plasmids encoding GFP (0.5 μg) (used as a marker for transfection with Bax) and 0.5 μg of pcDNA3 (control), pcDNA3-Bax, or pEGFP-Bak in combination with 1 μg (2-fold excess) of pcDNA3, pcDNA-3-Myc-Bcl-B, or pcDN3-FLAG-Bcl-X_L. At 24 h post-transfection, cells were collected and stained with DAPI. The percentage of GFP-positive cells with apoptotic morphology (fragmented nuclei or condensed chromatin) was determined (mean ± S.D.; $n = 3$). Immunoblotting of control cultures supplemented with 50 μM benzoyl-Val-Ala-Asp-fluoromethylketone to prevent apoptosis confirmed production of all proteins (also see Fig. 2). Similar results were obtained using HT1080 and PPC1 cells (not shown). *C* and *D*, HeLa cells were stably transfected with pcDNA3 (vector), pcDNA3-Myc-Bcl-B, or pRC-CMV-Bcl-2 plasmid and clones expanded. Representative data are presented showing two Bcl-B-transfected clones, clones 9 and 16. In *C*, immunoblot analysis was performed using lysates (20 μg of total protein) derived from control-transfected HeLa cells (vector), Bcl-B-transfected clones 9 and 16, and from HEK293T cells transiently transfected for 1 day with pcDNA3-Myc-Bcl-B. The blot was probed with anti-Myc antibody, followed by ECL-based detection. The bands corresponding to Myc-Bcl-B (*top*) and endogenous Myc protein (used here as a loading control) (*bottom*) are indicated. Note that the levels of Bcl-B produced in the stably transfected HeLa cell clones are considerably lower than levels of Bcl-B achieved by transient transfection of HEK293T cells. In *D* and *E*, HeLa transfectants were cultured in medium containing various concentrations of TRAIL (*D*) or STS (*E*) for 10 h. The percentage apoptotic cells was determined by DAPI staining (mean ± S.D.; $n = 3$). Symbols represent HeLa cells stably transfected with pcDNA3 parental plasmid (Neo) (*closed circles*), HeLa-Bcl-B (9) (*open squares*), HeLa-Bcl-B (16) (*closed squares*), and HeLa-Bcl-2 (*open circles*).

lower levels of Myc-Bcl-B, demonstrated only slight resistance to these apoptotic stimuli (Fig. 3, *D* and *E*). These data thus demonstrate that Bcl-B can suppress apoptosis induced by exogenous stimuli if expressed at sufficient levels. However, even HeLa cell clones with higher levels of Bcl-B did not manifest the profound resistance to apoptosis seen in Bcl-2-overexpressing cells (Figs. 3, *D* and *E*).

Many Bcl-2 family proteins associate with mitochondria in cells (reviewed in Refs. 1 and 2). Expression of GFP-tagged Bcl-B in cells revealed a punctate cytosolic pattern and partial co-localization with a mitochondria-specific dye (MitoTracker), as determined by two-color confocal microscopy (Fig. 4*A*). Crude subcellular fractionation analysis revealed that Myc-tagged Bcl-B protein resides predominantly in the mitochondria-containing HM fraction, similar to Bcl-2, as determined by immunoblot analysis of the cellular fractions (Fig. 4, *B* and *C*). In contrast to full-length Bcl-B, a truncation mutant of Bcl-B lacking the carboxyl-terminal TM domain (Bcl-BΔTM) targeted less efficiently to the HM fraction (Fig. 4*D*). The Bcl-BΔTM protein also was ineffective at blocking Bax-induced apoptosis (Fig. 4*E*), even though this protein was produced at comparable levels with the full-length Bcl-B protein. Thus, efficient organellar targeting appears to be required for optimal function of Bcl-B.

DISCUSSION

We describe a new member of the human Bcl-2 family protein, Bcl-B. This protein is most similar in amino acid sequence to the murine Boo (Diva) protein and the most similar among all human Bcl-2 family proteins to the CED9 protein of *Caenorhabditis elegans*. The Boo (Diva) protein interacts selectively with some Bcl-2 family proteins but not others, although controversy exists as to the details (9, 10). Interestingly, one report has suggested that the Boo protein can bind Bak but not Bax, and accordingly provided evidence that Boo suppresses apoptosis induced by overexpression of Bak but not Bax (10). Conversely, we observed that Bcl-B selectively binds and suppresses apoptosis induction by Bax, but fails to interact with or negate apoptosis triggered by Bak overexpression.

The murine Boo (Diva) protein has been variably reported to either suppress or promote apoptosis (9, 10). In transient transfection assays performed in four different human tumor cell lines, we consistently observed an anti-apoptotic action of Bcl-B. Stable overexpression of Bcl-B in HeLa cells also resulted in increased resistance to diverse apoptotic stimuli. However, because Bcl-B is capable of associating with either the anti-apoptotic proteins Bcl-2 and Bcl-X_L or with the pro-apoptotic protein Bax, it is possible that Bcl-B could display different phenotypes under some circumstances depending on cellular context. A similar phenomenon has been reported for some other Bcl-2 family proteins. For example, Bcl-2 can reportedly promote apoptosis in photoreceptor cells of the retina, while Bax can suppress cell death in some types of neurons

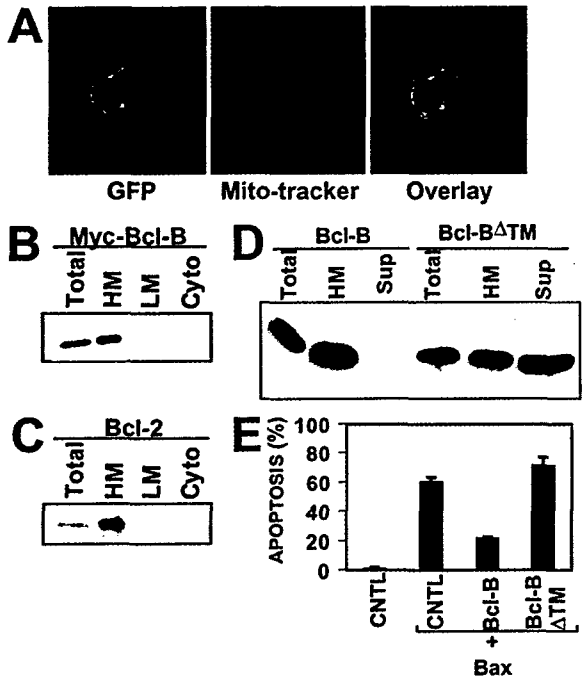

FIG. 4. **The COOH-terminal TM domain of Bcl-B is required for efficient membrane targeting and function.** *A*, for confocal microscopy analysis, a plasmid encoding GFP-Bcl-B was transfected into COS7 cells. At 24 h after transfection, cells were incubated with Mitotracker Red CMXRos, then fixed and imaged. Cells transfected with GFP control protein produced diffuse cellular fluorescence (not shown), in contrast to GFP-Bcl-B. *B–D*, for subcellular fractionation studies, HEK293T cells were transfected with plasmids encoding Myc-Bcl-B (*B*), Bcl-2 (*C*), and Myc-Bcl-B or Myc-Bcl-BΔTM (*D*). At 24 h post-transfection, cells were collected and post-nuclear lysates prepared (*Total*). An aliquot of these lysates was then fractionated by differential centrifugation at 10,000 × *g* to pellet HMs. The resulting supernatant (*Sup*) was then either analyzed directly (*D*) or subjected to centrifugation at 150,000 × *g* to pellet LMs and achieve a cytosolic supernatant (*B*, *C*). Proteins from each fraction were normalized relative to cell numbers and subjected to SDS-PAGE/immunoblot analysis using antibodies specific for Myc or Bcl-2. *E*, HEK293T cells were co-transfected with a plasmid encoding GFP (used as a marker for transfection) and either pcDNA3 (control (*CNTL*)) or pcDNA3-Bax, in combination with a 2-fold excess of pcDNA3 (control), pcDNA3-Myc-Bcl-B, or pcDNA3-Myc-Bcl-BΔTM. Cells were collected and stained with DAPI after 24 h. The percentage of *green* cells with apoptotic morphology was determined (mean ± S.D.; $n = 3$).

(11, 12).

Although stably transfected clones of HeLa cells, which contained higher levels of Bcl-B, exhibited resistance to exogenous apoptotic stimuli, the resistance afforded by Bcl-B was not as profound as that observed for Bcl-2 overexpression. This difference in potency of Bcl-B could be due to variations in the relative amounts of Bcl-B and Bcl-2 produced in transfected cells, or it could reflect a fundamental difference in the mechanisms of these proteins. In this regard, because Bcl-2 blocks cell death induced by both Bax and Bak, whereas Bcl-B inhibits apoptosis induced only by Bax but not Bak, it seems likely that Bcl-B may be less efficacious under circumstances where both Bax and Bak contribute to apoptosis induction. Bcl-B therefore may provide a mechanism for selectively inhibiting Bax-dependent apoptotic processes *in vivo*, while allowing Bak-dependent cell death to proceed normally.

The mouse Boo (Diva) protein was reported to associate with the caspase-activating Apaf1 protein (a homologue of *C. elegans* CED-4) (9, 10). Although we have observed weak interactions of Bcl-B with Apaf1 in co-immunoprecipitation assays, functional analysis has failed to reveal an effect of Bcl-B on Apaf1-induced apoptosis (not shown). Since Apaf1 is a soluble cytosolic protein (13), the inability of Bcl-BΔTM to suppress Bax-induced apoptosis also suggests that Bcl-B does not play a significant role in suppressing Apaf1. Moreover, the observation that Bcl-B suppresses apoptosis induced by Bax but not Bak also argues against a role for Bcl-B as an Apaf1 suppresser, given that both Bax and Bak induce mitochondrial release of the Apaf1 activator, cytochrome *c* (14, 15).

The correlation between membrane targeting and function is reminiscent of some other Bcl-2 family proteins and suggests that the site of action of Bcl-B is close to the intracellular organelles, including mitochondria, with which it associates. Although roughly half of the Bcl-BΔTM protein was associated with the HM membrane fraction in cells, this may be due to its dimerization with other resident Bcl-2 family proteins. A membrane site of action for Bcl-B would be consistent with evidence that several Bcl-2 family proteins are capable of forming ion channels or pores in membranes (reviewed in Ref. 16). Indeed, molecular modeling of Bcl-B on the structure of Bcl-X_L suggests that it possesses a similar overall fold and that it contains amphipathic α-helices similar to the putative pore-forming α5 and α6 of Bcl-X_L (not shown).

The differences observed in the functions and protein interaction partners of murine Boo and human Bcl-B proteins suggest that Bcl-B does not represent the human orthologue of mouse Boo/Diva. Also consistent with this interpretation is the difference in the patterns of expression of Bcl-B and Boo. Whereas Boo (Diva) is expressed predominantly in ovary, testis, and epididymis in adult mice (9, 10), RT-PCR analysis suggests that the *BCL-B* mRNA is widely expressed in adult human tissues. Comparisons of the sequence of *BCL-B* cDNAs with human genome sequence data indicate that the *BCL-B* gene is comprised of two exons interrupted by a ~2.3-kb intron. Interestingly, the location of this intron corresponds precisely to the intronic interruption in the coding region of the anti-apoptotic *BCL-2* and *BCL-X* genes (corresponding to the motif GGW^D or GGW/D in BH2 (see Fig. 1*B*). (The genomic sequence of murine *boo/diva* is unfortunately unavailable for comparison.) In contrast to *BCL-B*, the pro-apoptotic genes *BAX* and *BAK* have more complicated exon-intron organizations, in which the coding regions of the gene are spread over 5 (Bak) or 6 (Bax) exons. The similar genomic organization of the *BCL-2*, *BCL-XL*, and *BCL-B* genes thus suggests they evolved from a common ancestor and indirectly implies a similar mechanism of action for their encoded proteins.

Addendum—While this manuscript was in preparation, the cDNA sequence of Bcl-B was deposited into GenBank™ (accession number AF285092) by L. H. H. Zhang (unpublished data).

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